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14. ABSTRACT

In this project, we propose to use tumor-specific T cells to produce an immunotoxin (IT) targeting tumor cells only when these T cells are specifically activated by the tumor. We use lentiviral vectors to modify tumor specific T cells with our immunotoxin. PEA based immunotoxins affect cell viability by ADP ribozilation of their elongation factor-2. To produce high titer of vector encoding the IT we needed to generate a producer cell line resistant to PEA toxin. Several methods including the use of two mutated elongation factors, drug and peptides blocking PEA effect were used to protect the producer cells from the lethal activity of the toxin, thus increasing the production of proteins by these cells. We have established stable cell lines of PEA-resistant producer cells and showed that cells protected against PEA produced a significant higher amount of IT. Using these stable cell lines, we will produce a high titer of IT-lentivirus preparation. We have also generated vectors encoding survival genes to further protect the producer cells as well as the IT-modified tumor-specific T cells.

15. SUBJECT TERMS
Immunotoxin, tumor specific T cell, T cell activation, CD40L, antigen specific, Elongation factor

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INTRODUCTION.

Researchers have succeeded to enhance the effector function of monoclonal antibodies by coupling toxic moieties to the targeting portion of the antibody. These "warheads" have included radionucleotides and toxins (immunotoxins: IT). The antibody binds to the cell surface and the toxin is internalized into the cytosol, where it inhibits critical cell functions or damages the cell membrane, leading to cell death. The commonest IT are composed of the variable domain of a monoclonal antibody single (scFv) or double chain (dcFv), conjugated or fused via a linker sequence to a toxin that has been modified to decrease nonspecific binding to non-targeted normal cells. Although this approach has shown promise in animal models, toxicity issues have limited its clinical application. Only a small proportion of the drug reaches target cells after systemic injection, meaning that relatively high doses of IT are required to induce a significant biologic effect. Consequently, the increase in killing of target cells mediated by toxin is partly offset by increased toxicity. Non-specific clearance by liver and kidneys in particular may produce substantial and even fatal damage to these organs at doses optimal for anti-tumor activity. In hematological malignancies, for example, systemic injection of IT may cause vascular leak syndrome, thrombocytopenia, and liver damage. Here, we propose to minimize this toxicity by using tumor-antigen specific T cells to further target delivery of an immunotoxin, the *CD22-Pseudomonas exotoxin A* (CD22-PEA), which has already been used in a clinical setting. The toxin portion contains the translocating and ADP-ribosylating domains of PEA, and the native cell-binding portion is replaced with a CD22 scFv that directs targeting to B lymphocytes. CD22-PEA was tested in a Phase I trial in B-cell malignancies, but tumor responses, particularly in hairy cell leukemia, were offset by an unfavorable toxicity profile. The current project will use the anti-tumor activity of CD22-PEA while minimizing its adverse effects by delivery from T cells. Because the T cells we use are specific for tumoral antigen, they can only be activated when they encounter the tumor. By controlling the IT production with a promoter dependent on T cell activation, and using tumor specific T cells, we can limit the production and delivery of IT to the tumor site. This approach should increase the quantity of IT delivered to the tumor while preventing toxicity to the normal tissue.

BODY.

We have made significant progress on this project, having generated stable transduced 293T that express mutated elongation factor and having shown that they are resistant to the rapid killing effect of immunotoxins. More details results are reported below in the order described in the original S.O.W

Aim 1: To generate cytotoxic T lymphocytes (CTLs) specific for the tumor-associated antigens LMP1 and LMP2, and to engineer these lymphocytes to produce an anti-CD22-toxin following T cell activation (using CD40L promoter).

First year.

-**Construction of vectors**:

Integration of HA-tag in CD22-PEA vectors.

In order to trace our CD22-PEA protein more easily, we have inserted by PCR a Ha-Tag to the sequence encoding CD22-PEA. This tag will be used to trace the immunotoxin released by T cells following activation by tumor via the T cell receptor.

Anti-apoptotic vectors.

Numerous studies showed that cells that are highly resistant to apoptosis have relatively increased levels of telomerase activity. On the contrary, low expression of TERT is associated with vulnerability to apoptosis. We have generated lentiviral vectors encoding TERT. We have also shown that NQO1 induce the up-regulation of anti-apoptotic protein Bcl-2 levels thus providing a survival signal that protect cells from apoptosis. Our data from the T cells proliferation assay showed an advantage for the NQo1 transduced T cells. We have also generated lentiviral vectors encoding NQo1. These genes will be used to further protect 293 and T cells from the IT effect.

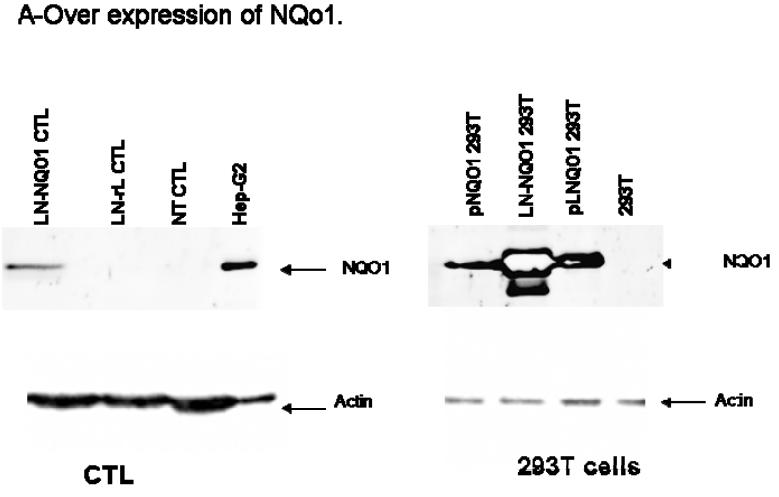


Figure 1: A- T and 293T cells were transduced with LN-NQO1 or a control LN vector, Hep-g2 cell were used as positive control for NQO1 expression. Western-blot showed that the protein was correctly overexpressed. B- An immuno-blot on NQO1- transduced T cells and parental T cells that were non-treated or treated to induce apoptosis (AP) (treatment down regulate anti-apoptosis genes) showed that the rescuing protein BCl-2 is induced in NQO1-modified cells (both treated and non-treated). Proliferation assay on T cells showed and advantage for the NQo1 transduced T cells.

Verify the vector constructions. We have verified the insertion of the genes in our expression plasmids and in our lentivirus vectors.

Transduction/selection of the 293T with mutated E2F

-Stable line 293T* and expansion.

PEA toxin kills cells by ADP-ribosilation of the elongation factor-2 which is essential for protein synthesis. This result in the decrease of protein produced in the cell and therefore leads to the death of the cell. To produce our IT, we need to protect 293T and rescue the activity of the elongation factor.
To rescue 293T cells from the IT, we have used a mutated elongation factor (mEF-2); the mutation created in this gene is located in the PEA targeted region of EF-2, it prevents the interaction of these 2 proteins and thus the cell death. We have cloned this mutated elongation factor in an expression vector and in a lentivirus plasmid also encoding a marker gene. The mEF-2-lentivirus particles were produced and concentrated by ultra-centrifugation, then used to transduce 293T cells. The marker gene was used to select transduced cells and to establish a stable line.

To the same end, we have also used a yeast mutant gene elongation factor (eEF2) which confers resistance to toxins (PEA and DT toxins). This later mutant was also inserted in a retroviral vector also encoding a puromycin resistance gene to inhibit the growth of non-transduced 293T cells and select a stable 293T line.

-Testing 293T * cultured with the recombinant toxin to ensure they are resistant.

-To test the proliferation and apoptosis of the transduce 293T cells, the parent and modified cells were transfected with IT-marker vector and monitored for several day. Our data revealed that transduced cells survived after exposition to the toxin.

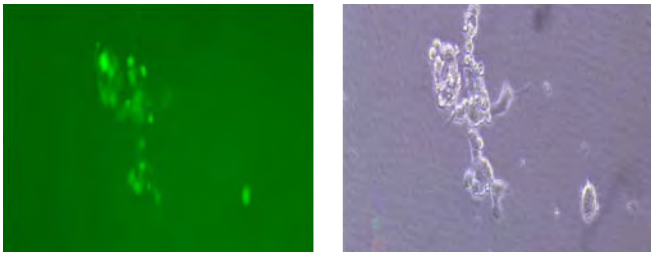


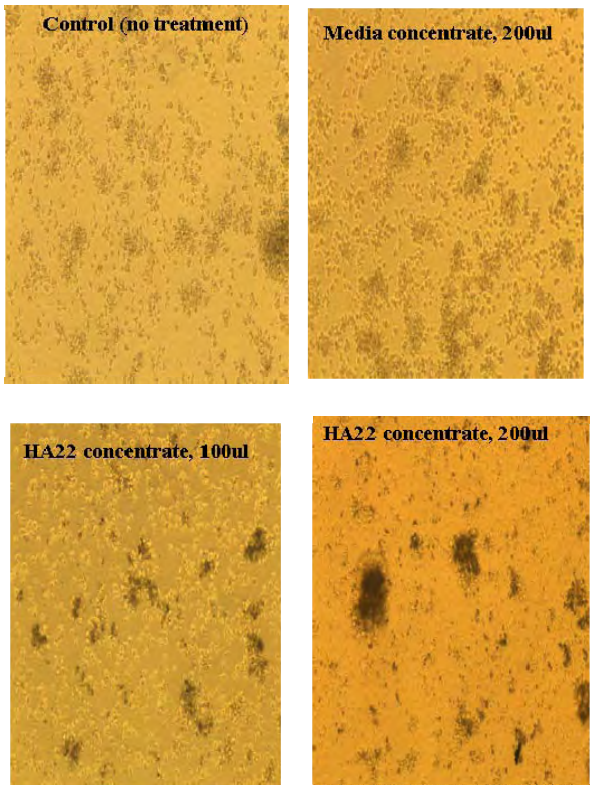
Figure 2: 293T cells were transfected with the vector expressing both the marker gene and the mEF-2, then exposed to the IT. Results showed that non-modified 293T cells died and marker$^+$ cell survived.

-A protein expression assay was also performed to test 293T resistance.
293T cell modified with either mutated elongation factor encoding vectors (these vectors only express a mutated EF-2, without a marker gene) was transduced with a plasmid encoding the green fluorescent protein (GFP). In these assay we expected the GFP to be expressed only if the elongation factor was not affected by the IT. Control cells were cells: 1-transduced with GFP only to detect the maximum expression, 2- non transfected. The highest GFP (+++) was in control GFP, the yeast EF-2 produced a very low level of GFP (+). However, as expected eukaryote mEF-2 produced a significant level of GFP (++).

In addition to the use of mutated elongation factor, we have used several approaches to protect 293T.

A-Meta-Iodobenzyguanidine (MIBG) is a high-affinity substrate for toxin that interferes with cellular monoADP-Ribosylation. We hypothesized that MIBG would prevents the toxin's interaction with EF-2 thus allowing cell survival.

Figure 3: Modified 293T-mEF stable (DMEM) were treated with MIBG 100mM (10ml/ml) and transfected (Promega calcium phosphate kit) with HA22 Plasmid. 48H after transduction, the CD22-PEA production was filtered using a .4μM filter and then concentrated. The production of CD22-PEA was then tested on target cells ($5x10^5$ cells/well), using 100 and 200 μl of 293T supernatant complete to 2ml with RPMI10% Serum. The cells were cultured for 3- 4 days then observed under microscope. Control consisted of regular culture media concentrate. We found that 293T supernatant was toxic to the LCL target cell (cluster of dead cells appeared brown).



B- HNP1, the human neutrophil protein 1 inhibited toxin mediated ADP-ribosylation of eEF2 (eukaryotic elongation factor 2). We have also investigated the use of this peptide and successfully detected the IT by immunoprecipitation. However because of the cost of this reagent and because we have found that its application in our project was not practical, we have discontinued these series of experiments.

C- To select toxin resistant 293T cells, these cells were cultured in presence of toxins for several weeks. We have obtained a stable cell line that grows in presence of toxic doses of the PEA toxin (determined as the dose which kills parental 293T). However when we have tried to produced the immunotoxin using these resistant cells we have had a poor production. We have reasoned that the resistant 293T were not sensitive to the PEA only because they down regulated the receptor or molecules involved in PEA cell entry. Productions of PEA directly within the cell compartments still harm the cell.

We have also investigated the use of SV40 pseudo virus, a non-viral vectors to directly deliver the DNA cassette containing CD40L promoter driving CD22-PEA) to T cells. *In vitro* packaging of DNA with SV40 capsid protein enables efficient delivery of plasmids and therefore would allow us to bypass the need of a producer cell line like 293T. Furthermore, SV40 pseudovirus has been used by our collaborator Dr Michael Gottesman to deliver immunotoxins in systemic (not directly at the tumor site). This vector was

optimized to modify T cells without affecting their viability (while keeping the expression of the transgene).

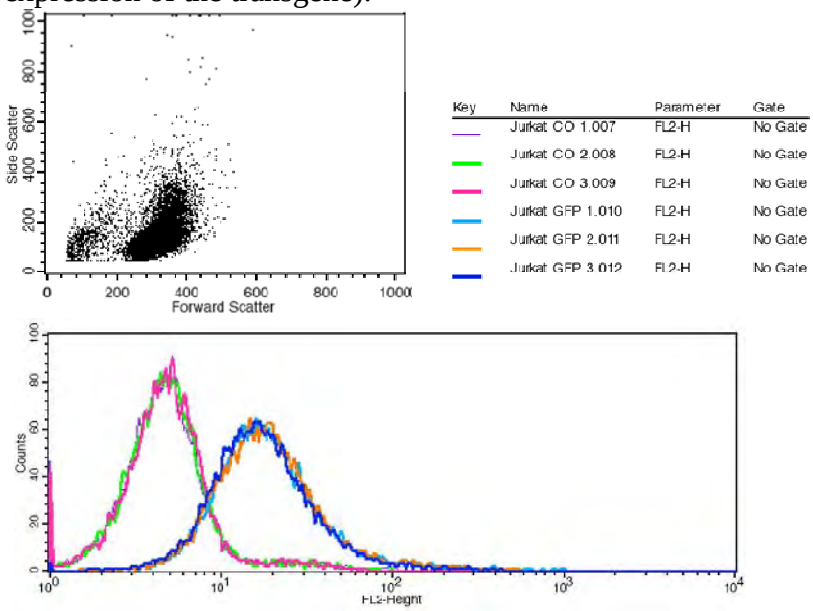


Figure 4-a: To evaluate SV40 pseudovirus transduction efficacy, Jurkat (T cells) were modified with the pseudovirus containing a GFP plasmid. Cells were analyzed by Flow cytometry 48h after treatment. Results show that Jurkat were efficiently modified.

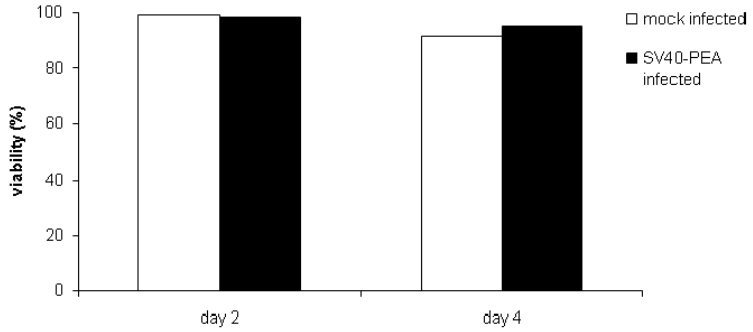


Figure4-b: The viability of Jurkat cells transduced by the pseudovirus was investigate using bleu trypan and showed that the transfection methods was not toxic to the T cells (Jurkat).

-Transduce 293T* with tag CD22-PEA.

-Western blotting on cell lysate and cell supernatants

We have investigated the ability of 293T carrying the mutated elongation factor-2 and the yeast to produce IT proteins despite the blocking effect of its PEA portion. 293T cells were modified with either elongation factors 24 hours prior the test (lane 1 and 3) or the day of the test (lane 4). These cells were then transduced with a plasmid encoding the GFP and with a vector containing the CMV promoter driving the IT. Controls consisted of parent-293T cells transduced with IT +GFP (lane 2) and parent-293T transduced GFP (lane 5). Cells were monitored for GFP expression. They were collected 48 hours after transfection and lysed. For each sample, equal amount of total proteins were separated by

SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane overnight at 4°C. The remaining steps were conducted according to a standard immunoblotting protocol. The results obtained showed that modified 293T produced the IT. The higher quantity of IT was produced when cells were protected by mammalian mEF-2 before the expression of the IT. GFP expression correlated with these finding, the higher expression been found for "24h mEF-2 293T+GFP". We feel that the yeast mutant may have not been fully efficient because all cultures were performed at 37°C.

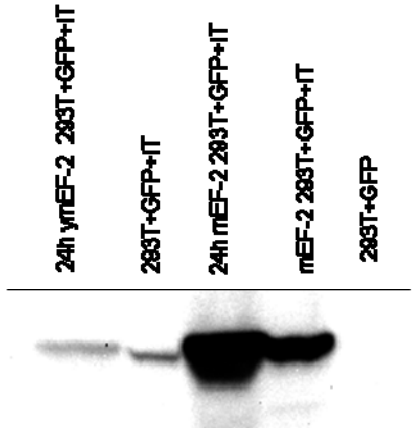


Figure 5: 293T cells modified with eukaryote or yeast mEF-2, GFP and IT vectors and lysed 48h later to detect the production of IT by western blot. Control cells were transduced with GFP only.

-IP (biologic activity test)/SDS page on cell supernatants.

Unprotected 293T cells were transduced with a vector encoding GFP and protected 293T cells with the IT. Concentrated cell supernatants were incubated with 10µg of PEA antibodies overnight at 4°C then incubated with protein G-agarose beads. The beads were washed with the lysis buffer described above three times and resuspended in protein sample buffer before the immunoprecipitation protein was subjected to immunoblotting. Blots were developed using the enhanced chemiluminesence procedure (Pierce Biotechnology, Inc., Rockford, IL) and images were acquired. Results showed that IT is produced by modified IT-transduced 293T (lane 1). No signal was obtained in control 293T cells. We have obtained similar data using 293T cells cultured in the presence of HNP-1 peptide

Figure 6: Supernatants from PEA-resistant 293T cells transfected with IT (lane1) and 293 GFP (lane) or were collected and used for an immunoprecipitation(IP). Immunoblot revealed that IT is present in the resistant 293T cultures supernatant.

-Production of viruses. The viruses production and testing is in progress

-Titration assays (p24 and FACS analysis)

- RCA

-Transduction of CTL lines. In progress

- We will use of 5 donors and 5 patients cells lines (confirm the feasibility the approach).

-Amplification of the transduced cell line. Not performed yet

KEY RESEARCH ACCOMPLISHMENTS.

- Generation of stable 293T cell lines cells that express a mutated elongation factor resistant to PEA toxin.
- Evidence that the elongation factor-modified 293T cells are resistant to lethal PEA toxin effect.
- Evidence that modified 293T produced immuntoxins.

REPORTABLE OUTCOMES.

-Development of cell lines:

Mutated-EF-2 transduced 293T stable cell line

Yeast EF-2 transduced 293T stable cell line

CONCLUSIONS.

We previously found that the production of IT was limited due to the effect of ADP-ribosylating domains of PEA. Therefore a protection from the IT is required to produce sufficient amount of IT-vector or IT proteins by 293T. We have found that yeast elongation factor was only partially protective since only a low amount of protein (both GFP and IT) was produced. This is probably related to temperature used of our culture. On the contrary, our second mutated elongation factor rendered 293T resistant to IT (cells were viable after been exposed to the IT and could produce green fluorescent proteins despite the presence of the IT). Thus, mEF-2 293T cells are allowing us to produce a higher quantity of protein and IT. This protection will allow us to increase the viral titer of our IT-lentivirus and therefore improve the transduction efficacy of our tumor–specific T cells. In this project we use T cells to directly and specifically deliver IT to the tumor cells. To insure the survival of IT-transduced T cells, these cells will also be transduced by our lentivector encoding the mutated elongation factor and/or our survival genes.

REFERENCES.

None